BINDING OF ENZYMES TO AVENA COLEOPTILE CELL WALLS[1, 2]

EUGENE F. JANSEN AND ROSIE JANG
WESTERN REGIONAL RESEARCH LABORATORY,[3] ALBANY, CAL.
AND
JAMES BONNER
CALIFORNIA INSTITUTE OF TECHNOLOGY,[4] PASADENA

It has been shown that the auxin, indoleacetic acid (IAA), exerts a marked influence on the pectic metabolism in growing cell walls of the Avena coleoptile (12). The methyl ester group in pectic substances is known to be derived, in part at least, from the methyl group of methionine (21, 22). Through the use of methionine-$C^{14}H_3$, it was shown that IAA markedly increases the rate of formation of the methyl groups in the cell wall pectic material without detectably changing the degree to which the pectic substances are esterified (12).

It has been suggested that the effect of IAA in promoting the elongation of cells is mediated through an effect on pectinesterase (PE). Some confusion exists as to whether IAA increases or effectively inhibits PE action. For example, Yoda (23) observed a parallelism between the effect of auxin on PE activity and on the water absorption of etiolated pea stems, and advanced the suggestion that the cell wall becomes elongated through a plasticization by PE activated by IAA. On the other hand, Glasziou (7, 8, 9) finds that IAA promotes the binding of PE to cell wall preparations from tobacco pith and artichoke tubers. He suggests that the immobilization of PE, by binding to the cell wall in vivo, favors methylation of the pectates of the cell wall and hence increases cell wall plasticity. However, he found the optimal concentration of IAA for binding of PE to cell wall preparations to be 10^{-9} M, a concentration far below that required to elicit the maximal rate of growth response. Conversely, Bryan and Newcomb (6) report that the PE activity of homogenates of tobacco pith is unaffected by IAA concentrations below 4×10^{-5} M but that concentrations of 1×10^{-4} M and higher completely inhibit the enzyme. They also observe that IAA increases the PE content of cultured tobacco pith just as it does the fresh weight.

The influence of IAA on PE activity and on the binding of PE to cell walls has therefore been reinvestigated to determine whether or not the effect of IAA in promoting the elongation of cells is mediated through an effect on PE.

MATERIALS AND METHODS

ENZYMES AND ASSAY METHODS: The PE was a relatively crude preparation from orange flavedo (15). It was dialyzed against 0.05 M $NaHSO_3$ at 5° C for several days, then against water until the sulfite had been removed. The preparation was then lyophilized. PE determinations were made by the standard procedure previously described (15). *a*-Chymotrypsin was prepared from chymotrypsinogen[5] recrystallized twice, dialyzed against water, and lyophilized. *a*-Chymotrypsin was assayed by a procedure similar to that for PE, with tyrosine ethyl ester as substrate (3).

A dialyzed and lyophilized preparation of the crystalline diisopropyl fluorophosphate inhibited *a*-chymotrypsin (10) was used in this study.

Crystalline pepsin prepared from commercial pepsin by the method of Northrop (19) was assayed for its rennet action (13).

Horseradish peroxidase[5] activity was measured by the method of Morris et al (18).

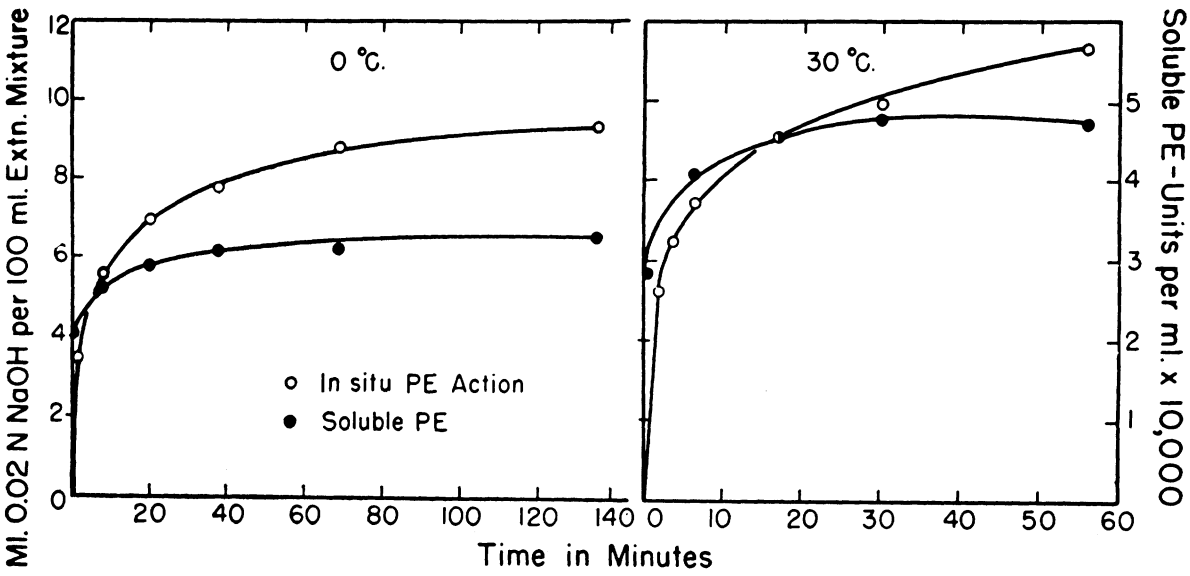
Nitrogen was determined with Nessler's reagent. Ammonium sulfate was used as a standard.

METHODS OF ANALYSIS: Pectic substance was measured by determining the uronic acid content using the pectinase-carbazole method of McComb and McCready (16) and McCready and McComb (17), expressing the pectic substance as anhydrouronic acid.

For determining methanol (methyl ester) content of cell wall preparations, 100 mg of dried samples was saponified with 4 ml of 1 *N* NaOH for 30 minutes at room temperature, and then 4.25 ml of 1 *N* HCl was added. The methanol so formed was distilled and determined by the method of Boos (5).

PREPARING AVENA COLEOPTILE CELL WALLS: Oat seeds of the variety Siegeshafer were germinated in Vermiculite moistened with distilled water, in stainless steel trays. The plants were grown at a temperature of 25° C and in low-intensity orange light until

[1] Received December 1, 1959.
[2] Work conducted under a cooperative agreement at the California Institute of Technology.
[3] A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.
[4] Division of Biology, California Institute of Technology.
[5] Purchased from the Worthington Biochemical Laboratory, Freehold, N. J.



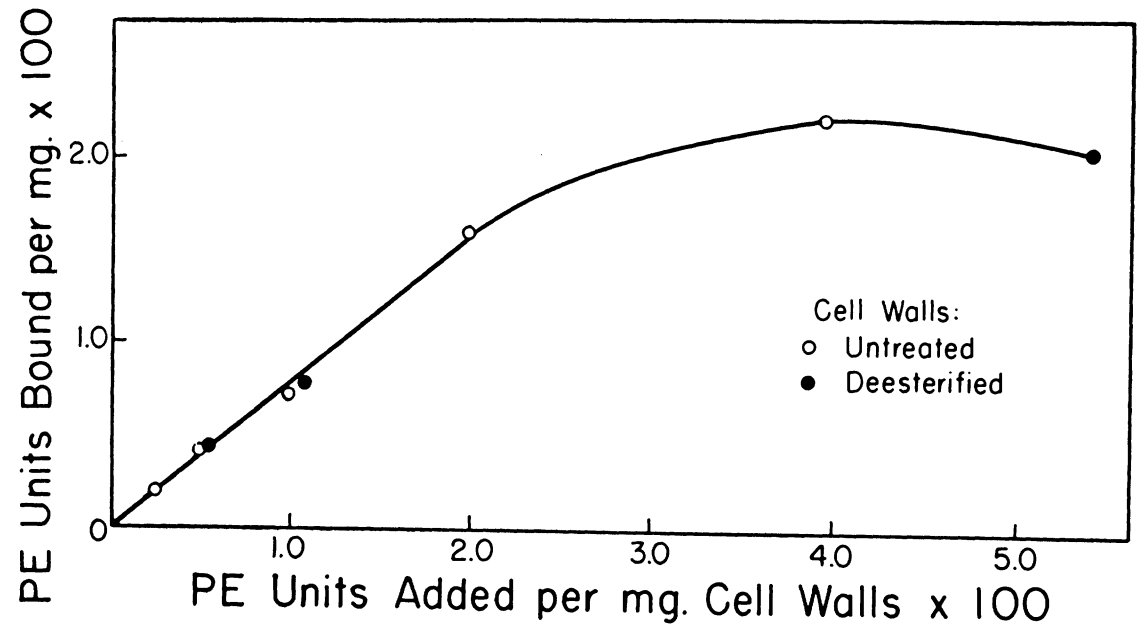


FIG. 1. PE action in situ and PE extraction at 0° C and 30° C: The action in situ was determined by measuring the amount of 0.02 *N* NaOH required to maintain pH 7.5 in a mixture of 50 ml of Avena coleoptile cell wall preparation plus 50 ml of H_2O and NaCl at a final concentration of 0.15 *N*. PE extraction was measured by periodic removal of aliquots which were then centrifuged and the PE activity of the supernatant determined.

FIG. 2. PE-binding capacity of untreated and deesterified cell walls: Weighed samples of partially purified PE were dissolved in 1 ml of cell wall suspensions at pH 5.6 (untreated contained 8.1 mg dry weight per ml and deesterified, 7.4 mg per ml) cooled to 0° C. After two hours the suspensions were centrifuged and aliquots of the supernatants were assayed for PE.

they had reached an average height of 3 cm (96 hrs). At this stage the seedlings were harvested.

Two methods of preparing cell walls were employed. One consisted of grinding the coleoptiles in an Omnimixer[6] at 0° C in four times their weight of water. The homogenate was centrifuged and the supernatant liquid decanted. The cell wall fraction was washed five times with water and finally suspended in water in a volume equal to twice the weight of the original coleoptiles. The pH of the suspension was 5.6.

Since PE bound to cell walls is inactive at pH values of 4.5 and lower (11), cell wall material was also prepared by grinding the coleoptiles in four times their weight of 0.15 M acetate buffer, pH 4.4 at 0° C. (PE assays of such homogenates, as well as the supernatant liquids obtained from centrifugation, showed that more than 90 % of the enzyme was still present in the cell walls.) Cell walls were then washed with water, and finally suspended in water as above. The pH of the final water suspension was 4.4.

Preparing Deesterified (PE-free) Cell Walls: Both the presence of salts and a pH of approximately 7.5 are known to be required for extraction of PE in orange tissue (15). The same conditions were also found to be necessary for extracting the enzyme from Avena coleoptile cell walls. The time course of the extraction of PE under these conditions and the simultaneous in situ PE action are given in figure 1 for temperatures of 0° and 30° C.

The major portion of the PE is extracted immediately upon adjustment of the pH of the extraction medium to 7.5. Further extraction of the enzyme occurs simultaneously with PE action in situ. These results appear to be in contrast to those obtained with orange cell walls. In the latter case, in situ PE action either preceded or occurred simultaneously with extraction of the enzyme. The results with orange suggest that the binding of PE to cell wall is similar to enzyme-substrate complex formation and that the enzyme is not extracted until the cell wall substrate has been hydrolyzed (11). Orange cell walls contain 39 % pectic substances (on a dry weight basis) of which 55 % is initially esterified (11) whereas coleoptile cell walls contain only 5.1 % pectice substances of which 43 % is esterified. Accordingly, less substrate is present for complexing in the latter cell walls and this may explain the difference in behavior of the two cell wall preparations.

Two types of deesterified Avena coleoptile cell wall preparations were made for enzyme binding studies; one preparation adjusted to pH 5.6 and the other to pH 4.4. For preparing the former, to 220 ml of a water-prepared cell wall suspension was added 1.93 g of NaCl and the suspension was cooled to 0° C, after which the pH was adjusted to 7.5 with 1 *N* NaOH. The suspension was stirred slowly and the pH maintained at 7.5 for 1.5 hours. Cell walls were then washed three times by centrifugation and resuspension in water. The suspension was adjusted to pH 5.6 with dilute HCl followed by three water washes and finally suspended in water to a total volume of 220 ml. Deesterified cell walls at pH 4.4 were similarly prepared.

The composition of untreated and deesterified cell wall preparations, both suspensions at pH 4.4, are given in table I.

Preparing Tobacco Pith Homogenate: A 245 mm section of the stem above the fourth node of a 3.5 ft plant was used for this purpose. The pith was removed and cut into 3 mm sections. To the sections was added 7 ml of 0.1 M phosphate buffer (pH 7.0) and the mixture was ground in an Omnimixer[6] at 0° C.

Removal of Pectic Material from Cell Walls with Purified Polygalacturonase: For the specific removal of pectic material from Avena cell walls, to 50 ml of deesterified cell wall preparation (0.55 g dry wt of cell walls) were added 20 ml of 1 M acetate buffer at pH 5.0, 20 ml of 0.5 % ethylenediaminetetraacetate, 10 ml of H_2O, and 20 mg of highly purified polygalacturonase (0.40 polygalacturonase unit) (14). The reaction mixture was stirred slowly for 3 hours at 25° C, toluene was added as a preservative, and the mixture allowed to incubate for 6 days. Analysis of the supernatant by the method of McComb and McCready (16) showed that approximately 90 % of the cell wall pectic material had been extracted. This result was confirmed by analysis of the cell walls [by the method of McCready and McComb (17)] after adjusting the pH of the reaction mixture to 4.4, centrifuging, and washing the cell walls five times with water. Volume of the final suspension in water was 50 ml. Supernatant and washings from the polygalacturonase treatment

Table I

Composition of Avena Coleoptile Cell Wall Preparations

	Untreated	Deesterified
Dry wt, %[1]	0.81	0.74
Pectic material %[2]	5.1	5.5
CH_3OH, %[3]	0.40	0.03
Molar ratio CH_3OH/AUA	0.43	0.03
PE units/g[4]	0.11	0

[1] Dry weight of the aqueous suspension of cell wall preparation.

[2] Anhydrouronic acid on dry weight basis.

[3] For CH_3OH determinations the cell wall preparations were centrifuged, washed several times with acetone by suspension and recentrifugation, and then dried in vacuo. Results are reported on a dry weight basis.

[4] On dry weight basis.

[6] Mention of trade names, products or manufacturers does not imply endorsement by the U. S. Department of Agriculture over others not mentioned.

were combined and concentrated in vacuum. Through the use of ion-exchange resins and paper chromatography the only monosaccharides which were liberated from the cell walls by polygalacturonase were glucose and galacturonic acid.

The preparation of commercial pectinase treated cell walls, which resulted in the removal of other polysaccharides in addition to the pectic material, has been reported earlier (12).

Results

PE-binding Capacity of Cell Walls: The PE-binding capacities of untreated and deesterified cell walls were determined with partially purified PE preparation (fig 2). Assays of the supernatants showed that 80 % of the PE added, up to a concentration of 0.02 PE unit per mg of cell wall, was adsorbed by the cell walls. Untreated and deesterified cell walls behaved identically. The maximum amount of PE bound was 0.022 PE unit per mg of cell wall, an amount 200 times greater than the native PE content of cell walls. The adsorbed PE was firmly bound since repeated water washing failed to elute any of the enzyme. The bound PE could be completely eluted at pH 7.5 in the presence of 0.15 *N* NaCl. For example, when 0.01 PE unit of enzyme was added per mg cell wall, assays of the supernatant showed that 0.008 PE unit was bound. The cell walls were centrifuged and the pellet washed five times by suspension in water and centrifugation. The cell walls were then suspended in 0.15 *N* NaCl (7.4 mg of cell walls/10 ml of solution), the pH adjusted to 7.5 and after 15 minutes again centrifuged. Assays of the supernatant showed that 0.0082 PE unit had been eluted. Thus all of the bound PE was recovered by elution. Similarly, when cell walls which had been treated with 0.02 PE unit per mg and which had bound 0.016 unit per mg were water washed and extracted as above, 0.016 PE unit was eluted. Therefore, water washing failed to remove PE but the enzyme was completely eluted at pH 7.5 in the presence of 0.15 *N* NaCl. These results suggest that the binding of PE to cell walls is simply an ionic binding.

Absence of Effect by IAA on PE-binding Capacity of Cell Walls or PE Activity: Since it had been reported that IAA promotes the binding of PE to cell wall homogenates (7, 8, 9), the effect of IAA on the PE binding capacity of Avena coleoptile cell walls has been determined. The results are presented in table II. It is clear that IAA is without effect on the PE-binding capacity of cell walls. The amount of PE bound in the presence and absence of IAA was determined by elution of the enzyme from the water washed cell walls subsequent to binding. PE assays of the supernatants from the original adsorption mixture after two hours at 0° C agreed with the elution data. Since the PE-binding capacity of cell walls is large and since the binding is apparently ionic, it is not surprising that it is not influenced by IAA.

Table II

PE Bound to Cell Walls[1] in Presence and Absence of IAA[2]

IAA	PE Units added/mg cell wall × 100[3]	PE Units bound[4]/mg cell wall × 100
Absent	2.5	1.8
Present	2.5	1.8

[1] Deesterified preparation of Avena coleoptile cell walls at pH 4.4 was used.
[2] The concentration of IAA in the adsorption mixture was 1×10^{-4} M.
[3] On dry weight basis.
[4] As determined by elution of bound PE.

The effect of IAA on PE activity was re-examined using tobacco pith homogenates as the enzyme source. The homogenate contained 0.0042 PE unit per ml. When the homogenate was assayed in the presence of 1×10^{-4} M IAA (added to the substrate prior to adding the homogenate), an identical activity of 0.0042 PE unit per ml was recovered. The homogenate possessed an apparent PE content of 0.0031 unit per ml when the substrate was 0.06 M with respect to $CaCl_2$ rather than the usual 0.15 *N* with respect to NaCl. In the presence of 1×10^{-4} M IAA, an activity of 0.0030 PE unit per ml was found. Therefore, under the conditions investigated, IAA was without a detectable effect on PE activity. These results are contrary to those reported by Bryan and Newcomb (6). The reason for the difference is not apparent.

Binding Capacity of Cell Walls for Pepsin, α-Chymotrypsin, and Peroxidase: Avena coleoptile cell walls, both untreated and deesterified preparations, are capable of firmly binding large amounts of pepsin, α-chymotrypsin, and peroxidase. The binding of these enzymes to cell walls is illustrated in figure 3. With α-chymotrypsin the maximum amount bound was 0.85 mg of enzyme per mg (dry wt) of deesterified cell wall preparation. At concentrations of 0.6 mg per mg of cell walls or less, essentially all of the α-chymotrypsin was bound. The maximum binding capacity of esterified cell wall preparation was approximately 80 % that of the deesterified preparation. Like PE, α-chymotrypsin was firmly bound and remained on the cell walls after washing with water but could be eluted with 0.15 *N* NaCl at pH 7.5.

Even larger amounts of pepsin are bound to cell walls. At the lower levels of pepsin concentration, essentially all of the added enzyme was bound. Not shown in figure 3 is the fact that when 2.27 mg of pepsin was added per mg cell walls, 2.02 mg (89 %) was bound. The amount of pepsin bound of necessity was determined by assay of the supernatant because of the extreme instability of pepsin at pH 7.5 (20). Water washing of cell walls failed to remove any of the bound pepsin. Cell walls, which had bound 1.27 mg of pepsin and had subsequently been washed with

water were treated with 0.15 *N* NaCl solution at pH 6.0 [a pH at which pepsin is reasonably stable (20)]. This resulted in the elution of 58 % of the bound pepsin.

That the binding of enzymes to Avena coleoptile cell walls is apparently not a function of the isoelectric point of the enzyme is suggested by the data for pepsin and α-chymotrypsin. The isoelectric point of the former is pH 2.7 (20) and that of the latter pH 8.6 (2).

Peroxidase was also firmly bound to cell walls but the binding capacity was less than for proteolytic enzymes. The results in figure 3 were obtained by elution of the peroxidase from water-washed, peroxidase-treated cell walls. The elution procedure was the same as that used for PE. With peroxidase, the

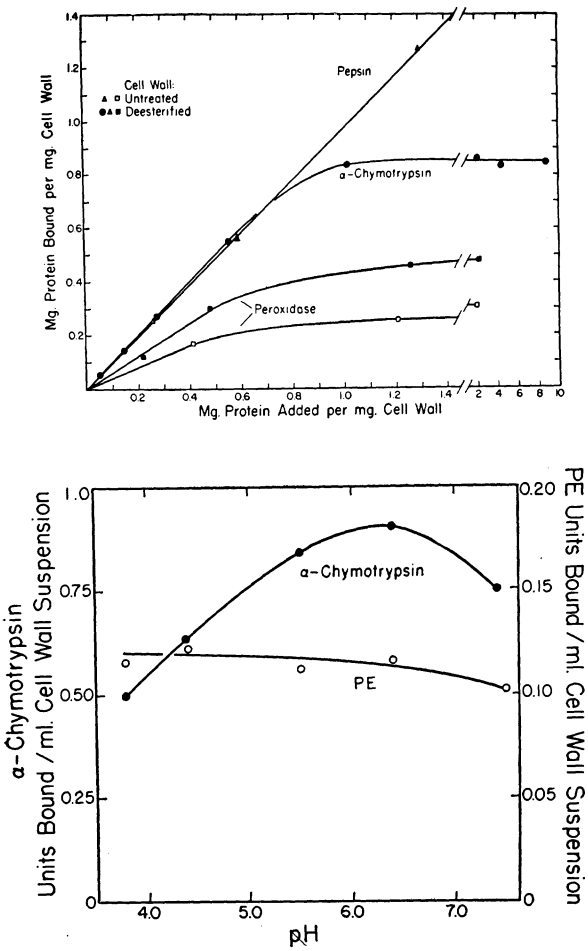


FIG. 3. Binding of pepsin, α-chymotrypsin and peroxidase to cell walls: Weighed samples of enzyme were dissolved in 1 ml of cell wall suspensions, both untreated and deesterified, and incubated for 2 hours at 0° C after which the suspensions were centrifuged and enzyme binding determined (see text). For binding α-chymotrypsin, cell wall suspensions at pH 5.6 were used. For the pepsin and peroxidase binding, suspensions at pH 4.4 were used.

FIG. 4. Effect of pH of cell walls on enzyme binding. (see text).

binding capacity of the cell walls is markedly increased by deesterification of their pectic substances. The maximum binding capacity of the untreated cell walls was 0.31 mg peroxidase per mg cell walls while that of the deesterified cell walls was 0.48 mg per mg. The ratio of binding capacity of deesterified/untreated cell walls, 1.55, is in reasonable agreement with the ratio of free carboxyl groups of the pectic substances of the two types of cell walls, 97 %/57 % or 1.67 (table I.). These results suggest that peroxidase may be bound to the free carboxyl groups of the pectic substances of the cell walls. Since larger amounts of the proteolytic enzymes are bound to cell walls, other binding sites must also be involved. This will be shown below to be the case.

Heating of cell walls had no effect on their PE binding capacity. To a deesterified cell wall preparation at pH 4.4 which had been heated in boiling water for five minutes and then cooled, was added 0.02 PE unit per mg cell walls. After incubating, centrifugating, water washing, and eluting the cell walls, 0.015 PE unit per mg was found to have been bound. This value is in good agreement with the comparable one of figure 2 (0.016). Similar results were obtained for binding α-chymotrypsin by heated cell walls.

Ethylenediaminetetraacetate at pH 6.0 neither altered the binding capacity of cell walls nor dissociated bound enzyme. Deesterified cell walls, pretreated with 1.8 mg of α-chymotrypsin per mg cell walls, was extracted with 0.1 % ethylenediaminetetraacetate solution at pH 6.0 for 1 hour. The supernatant contained only 6 % of the bound enzyme. Untreated cell walls at pH 4.4 (2 ml of suspension) were stirred with 10 ml of 0.5 % ethylenediaminetetraacetate at pH 6 for 3 hours. To these cell walls, after washing with 0.15 M acetate buffer at pH 4.4 and with water, was added 1 mg of α-chymotrypsin per mg of cell walls. The amount of enzyme bound as determined by the amount eluted was 0.64 mg per mg cell walls. This amount was the same as that of untreated cell walls at this pH (see fig 4). Therefore calcium or similar cations are not involved in binding enzymes to cell walls.

Living Avena coleoptile sections themselves do not bind PE. Sections (12 mm long) were prepared by the method used previously (12). To 0.5 ml of H_2O solution containing 0.16 PE unit was added 270 mg of sections and the mixture incubated for 2 hours at 0° C. PE assays of the solution showed that it still contained 0.16 PE unit. These results suggest that the cells must be ruptured in order to expose the PE binding sites of the cell walls.

EFFECT OF PH OF CELL WALLS ON ENZYME BINDING: To determine the effect of the pH of cell walls on their enzyme binding, 2.5 ml aliquots of water-washed deesterified cell walls at pH 4.4 were suspended in 10 ml of 0.15 M acetate buffers of pH 3.8, 4.4, 5.5, 6.4, and 7.5. The cell walls were centrifuged and washed five times with water. Each sample was then suspended in water to give the original volume of 2.5

ml. For determining the effect of pH on the binding of α-chymotrypsin, to 1 ml suspension of each was added 0.425 α-chymotrypsin unit (8 mg enzyme/ml or 1.08 mg/mg of cell walls) and for the effect on PE binding, 0.16 PE unit per ml (0.0216 PE unit per mg). After 1 hour at 0° C the cell walls were centrifuged and the supernatants assayed for α-chymotrypsin or PE. Each was then water washed and eluted as above. The results reported (fig 4) were those obtained by assays of the eluted enzymes which agreed well with those obtained by assays of the supernatants. The pH of the cell walls had little effect on PE binding (fig 4), but the pH of the cell walls markedly affected α-chymotrypsin binding with maximal binding occurring at pH 6.4 and minimal at pH 3.8. The difference observed in the effect of pH on the binding of these two enzymes suggests that they are bound to different sites on the cell walls.

DIFFERENT SITES FOR BINDING PROTEINS TO CELL WALLS: That there is more than one type of protein binding site on Avena coleoptile cell walls was established in several ways. The results (table III) show that the saturation of cell walls with α-chymotrypsin does not change the binding capacity of the preparation for PE and vice versa. Adding the second enzyme does displace (or exchange with) 20 to 25 % of the first enzyme used for saturation of the cell walls. The results clearly demonstrate that these two enzymes are principally bound to separate sites on cell walls. The limited displacement of the first enzyme by the second may possibly represent a third common site.

Further evidence of multiplicity of binding sites was obtained with the use of diisopropyl fluorophosphate inhibited α-chymotrypsin. The inhibited enzyme was added to deesterified cell walls at a concentration of 1.1 mg per mg cell walls and incubated for 2 hours at 0° C after which the cell walls were water washed and eluted as with those treated with the active enzyme. The results (table IV) show that the inhibited enzyme was firmly bound to cell walls in an amount 73 % that of the active enzyme. Cell walls saturated with the inhibited enzyme still bind 62 % of the amount of active enzyme bound by cell walls not so treated. Apparently, the active and inhibited α-chymotrypsin are largely bound to different sites.

Previous results demonstrated that binding PE and α-chymotrypsin to orange cell walls involved the pectic material of these cell walls since the specific removal of pectic substances therefrom with highly purified polygalacturonase (14) resulted in a proportional loss in the binding capacity for both enzymes (11). This was not the case, however, with Avena coleoptile cell walls where specifically removing 90 % of the pectic material of the cell walls decreased their binding capacity for PE and α-chymotrypsin by only

TABLE IV

BINDING OF α-CHYMOTRYPSIN AND ITS DIISOPROPYL FLUOROPHOSPHATE INHIBITION PRODUCT TO CELL WALLS[1]

CELL WALL TREATMENT	MG PROTEIN BOUND/MG CELL WALL[2]	
	α-CHYMOTRYPSIN	INHIBITED CHYMOTRYPSIN
None	0.56[3]	...
None	...	0.41[4]
Saturated[5] with inhibited chymotrypsin	0.35[3]	...

[1] Deesterified cell wall preparation at pH 4.4 was used.
[2] On dry weight basis.
[3] As determined by elution of the bound enzyme.
[4] As determined by N analysis of eluted protein.
[5] Cell walls were washed four times with H_2O by suspension and centrifugation after adsorption of the inhibited enzyme.

TABLE III

BINDING OF PE AND α-CHYMOTRYPSIN TO DIFFERENT SITES ON CELL WALLS [1]

CELL WALL TREATMENT	UNITS OF ENZYME/MG CELL WALL × 100[2]					
	ADDED		EXCHANGED[3]		BOUND[4]	
	PE	α-CHYMOTRYPSIN	PE	α-CHYMOTRYPSIN	PE	α-CHYMOTRYPSIN
None	2.2	...	...	...	1.3	...
None	...	5.7[6]	...	...	...	2.9
PE-saturated cell walls[5]	...	5.7[6]	0.23	...	1.1	3.0
α-Chymotrypsin saturated cell walls[5]	2.2	...	...	0.74	1.2	2.2

[1] Deesterified cell wall preparation at pH 4.4 was used.
[2] On dry weight basis.
[3] As determined by assay of supernatant after adding second enzyme.
[4] As determined by eluting the bound enzymes.
[5] Cell walls were washed four times with H_2O by suspension and centrifugation after the adsorption of first enzyme.
[6] 1.08 mg α-chymotrypsin per mg cell wall.

TABLE V

EFFECT OF POLYGALACTURONASE AND PECTINASE ON BINDING CAPACITY OF CELL WALLS[1]

CELL WALL TREATMENT	PROTEIN BOUND, %[2]		
	PE	α-CHYMOTRYPSIN	INHIBITED CHYMOTRYPSIN
None	100	100	100
PG	77	70	...
Pectinase	17	13	35

[1] Deesterified cell wall preparation at pH 4.4 was used.
[2] As determined by elution of the bound proteins.

23 and 30 %, respectively (table V). Accordingly, the pectic material of the cell walls plays only a minor part in binding these two enzymes. Removing all the pectic material together with other polysaccharides by commercial pectinase decreased the binding capacity of the cell walls for PE and α-chymotrypsin to approximately 15 % of the original value. On the other hand, 35 % of the binding capacity for inhibited chymotrypsin remained in cell walls so treated. These results clearly indicate the existence of more than one type of protein binding site in Avena cell walls.

EFFECT OF INCUBATING AVENA COLEOPTILE SECTIONS AT PH 4.4 AND PH 7.4 ON PE ACTION IN SITU: Auxin-induced elongation of Avena coleoptile sections proceeds at maximal rate at pH 4.4 and much more slowly at pH 7.4 (4). If the effect of auxin were mediated through binding of PE, then this binding should be maximal at pH 4.4 and PE action in situ should be maximal at pH 7.4. In order to obtain additional evidence on the auxin-PE binding hypothesis, sections were incubated in solutions at these two pH values. Avena coleoptile sections were prepared by the method previously used (12). One 20 g lot was added to 1 liter of 0.75 M phosphate buffer at pH 7.4 and another to 1 liter of 0.15 *N* acetate buffer at pH 4.4. Both were incubated for 18 hours with occasional stirring. After water washing, each was homogenized at 0° C with 80 ml of 0.15 *N* acetate buffer at pH 4.4. Assays of the homogenates showed that the sections incubated at pH 4.4 contained 0.0018 PE unit per g fresh weight and those incubated at pH 7.4, 0.0016 PE unit per g. The cell walls were centrifuged, water washed five times, and finally suspended in a total volume of 40 ml of water. The cell walls contained respectively 0.0014 and 0.0015 PE unit per g of original section. The cell walls were next filtered, washed with acetone, and then dried in a vacuum desiccator. The pectic substance content of each cell wall preparation was 5.1 % and the CH_3OH content was 0.42 % for cell walls prepared from sections incubated at pH 4.4; 0.36 % for sections incubated at pH 7.4. The pectic substance content was the same as that of cell walls prepared from non-incubated sections (table I). The lower CH_3OH content in cell walls from sections incubated at pH 7.4 indicates a slight in situ PE action during incubation at the higher pH value. The fact that the pH at which incubation was conducted does not influence the amount of PE accompanying the cell walls as well as the fact that pH 7.4 incubation resulted in only a trace of in situ PE action are both at variance with expectations of the auxin-PE binding hypothesis.

DISCUSSION

In the present investigations, no evidence has been obtained to support the suggestion that the effect of IAA in promoting the elongation of cells is mediated through an effect on PE. Avena cell walls are capable of firmly binding added PE in an amount approximately 200 times greater than that inherent to the cell walls. The binding is apparently ionic since all of the bound enzyme can be eluted with salt solutions at pH 7.5. IAA does not influence the binding of PE to cell walls nor does it affect the PE activity of tobacco pith homogenates. These results are not in accord with previous reports (6, 7, 8, 9, 23). Avena cell walls are also capable of firmly binding other proteins such as pepsin, peroxidase, α-chymotrypsin and its diisopropyl fluorophosphate inhibition product in large amounts. These proteins are likewise ionically bound. In other words, Avena coleoptile cell walls have much in common with certain chromatographic columns. On the other hand, living, intact Avena coleoptile sections do not bind added PE. Therefore the suggestion that IAA exerts its effect through a specific binding of PE is not tenable.

Previous results have shown that IAA does affect the pectic metabolism of the growing Avena coleoptile section (12). IAA increases the formation of the methyl ester of the hot-water-soluble portion of the pectic material of the cell wall without causing a detectable change in the degree of esterification of any of the pectic material of the cell walls. IAA causes an even greater increase in the rate of formation of methyl ester in the cold-water-soluble pectic material (12). Furthermore, IAA causes the same increase in the formation from glucose of the galacturonic acid residues of these two pectic fractions (1). This was demonstrated by incubating sections in the presence of C^{14}-labeled glucose with and without IAA, followed by isolation of the galacturonic acid and determination of its radioactivity. Whether or not the effect of IAA on the pectic metabolism of sections is its primary effect in promoting the elongation of cells remains to be proven. The fact that no IAA-induced net change in the degree of esterification of any of the pectic material of the cell walls could be detected constitutes additional evidence against the auxin-PE binding hypothesis.

SUMMARY

I. Aqueous suspensions of Avena coleoptile cell walls are capable of firmly binding added pectinesterase in amounts up to approximately 200 times that native to the cell walls. This is true for both native cell walls and walls in which the pectic methyl ester groups have been removed by pectinesterase. The

enzyme remained bound through exhaustive water washing but is readily eluted with 0.15 *N* NaCl at pH 7.5. The binding is apparently an ionic one.

II. The pectinesterase-binding capacity of cell walls is not influenced by added indoleacetic acid. The auxin is also without effect on the pectinesterase activity of tobacco pith homogenates.

III. Large amounts of pepsin, peroxidase, α-chymotrypsin and its diisopropyl fluorophosphate inhibition product are similarly firmly bound to Avena coleoptile cell walls. As much as 0.85 mg of α-chymotrypsin and 2.0 mg of pepsin are bound per mg of cell walls. These proteins are elutable by the method used for pectinesterase.

IV. Neither heating of cell walls in suspension nor treatment with ethylenediaminetetraacetate changes their binding capacity for pectinesterase or α-chymotrypsin. This sequestering agent also fails to liberate the bound enzymes.

V. Intact Avena coleoptile sections do not bind added pectinesterase.

VI. The pH of cell walls over the range of pH 3.8 to 7.5 does not influence their binding capacity for pectinesterase. The binding capacity for α-chymotrypsin is maximal at pH 6.4.

VII. Pectinesterase and α-chymotrypsin are bound to different sites in the cell wall since saturation with one does not influence the binding of the other. Saturation of cell walls with diisopropyl fluorophosphate-inhibited α-chymotrypsin decreases the binding capacity for α-chymotrypsin by 40 %.

VIII. The specific removal of pectic material from cell walls by highly purified polygalacturonase results in only partial reduction in the binding capacity for pectinesterase and α-chymotrypsin. Cell walls treated with pectinase, which removes polysaccharides in addition to pectic material, still retain approximately 15 % of their binding capacity for these two enzymes.

IX. Incubation of Avena coleoptile sections at pH 4.4 where auxin is most effective and at pH 7.4 where it is but slightly effective showed no difference in the amount of pectinesterase accompanying the cell walls or any significant difference in the degree of esterification of the pectic material in the cell walls.

X. No evidence has been obtained to support the view that the effect of auxin in promoting the elongation of cells is mediated through an effect on pectinesterase.

Acknowledgment

The technical assistance of Mr. Dennis Barrett during part of these investigations is gratefully acknowledged.

Literature Cited

1. Albersheim, P. and J. Bonner. 1959. Metabolism and hormonal control of pectic substances. Jour. Biol. Chem. 234: 3105–3108.
2. Anderson, E. A. and R. A. Alberty. 1948. Homogeneity and the electrophoretic behavior of some proteins. II. Reversible spreading and steady-state boundary criteria. Jour. Phys. and Colloid Chem. 52: 1345–1364.
3. Balls, A. K. and E. F. Jansen. 1952. Stoichiometric inhibition of chymotrypsin. Advances in Enzymol. 13: 321–343.
4. Bonner, J. 1934. The relation of H ions to the growth rate of Avena coleoptiles. Protoplasma 21: 406–423.
5. Boos, R. N. 1948. Quantitative colorimetric determination of methanol with chromotropic acid reagent. Anal. Chem. 20: 964–965.
6. Bryan, W. H. and E. H. Newcomb. 1954. Stimulation of pectinesterase activity of cultured tobacco pith by indoleacetic acid. Physiol. Plantarum 7: 290–297.
7. Glasziou, K. T. 1957. The effect of 3-indolylacetic acid on the binding of pectin methylesterase to the cell walls of tobacco pith. Aust. Jour. Biol. Sci. 10: 337–341.
8. Glasziou, K. T. 1957. The effect of auxins on the binding of pectin methylesterase to cell wall preparations. Aust. Jour. Biol. Sci. 10: 426–434.
9. Glasziou, K. T. and S. D. Inglis. 1958. The effect of auxins on the binding of pectin methylesterase to cell walls. Aust. Jour. Biol. Sci. 11: 127–141.
10. Jansen, E. F., M-D. F. Nutting, R. Jang, and A. K. Balls. 1949. Inhibition of the proteinase and esterase activities of trypsin and chymotrypsin by diisopropyl fluorophosphate: Crystallization of inhibited chymotrypsin. Jour. Biol. Chem. 179: 189–199.
11. Jansen, E. F., R. Jang, and J. Bonner. 1960. Orange pectinesterase binding and activity. Food Res. 25: 64–72.
12. Jansen, E. F., R. Jang, P. Albersheim, and J. Bonner. 1960. Pectic metabolism of cell walls. Plant Physiol. 35: 87–97.
13. Kunitz, M. 1935. A method for determining the rennet activity of chymotrypsin. Jour. Gen. Physiol. 18: 459–466.
14. Lineweaver, H., R. Jang, and E. F. Jansen. 1949. Specificity and purification of polygalacturonase. Arch. Biochem. 20: 137–152.
15. MacDonnell, L. R., E. F. Jansen, and H. Lineweaver. 1945. The properties of orange pectinesterase. Arch. Biochem. 6: 389–401.
16. McComb, E. A. and R. M. McCready. 1952. Colorimetric determination of pectic substances. Anal. Chem. 24: 1630–1632.
17. McCready, R. M. and E. A. McComb. 1952. Extraction and determination of total pectic materials in fruits. Anal. Chem. 24: 1986–1988.
18. Morris, H. J., C. A. Weast, and H. Lineweaver. 1946. Seasonal variation in the enzyme content of eleven varieties of carrots. Bot. Gaz. 107: 362–371.
19. Northrop, J. H. 1946. Crystallization of pepsin from alcohol. Jour. Gen. Physiol. 30: 177–184.
20. Northrop, J. H., M. Kunitz, and R. M. Herriott. 1948. Crystalline enzymes. 2nd Ed. Columbia University Press, New York.
21. Ordin, L., R. Cleland, and J. Bonner. 1955. Influence of auxin on cell wall metabolism. Proc. Nat. Acad. Sci. 41: 1023–1029.
22. Sato, C. 1953. Methyl group synthesis in plant metabolism. Thesis. Michigan State University.
23. Yoda, S. 1958. Effect of auxin on pectic enzymes. Bot. Mag. Tokyo 71: 1–6.